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Comparisons of diverse plant species reveal that only grasses show drastically reduced levels of ubiquitin monomer in mature pollen

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Abstract Ubiquitin is a ubiquitous protein involved in targeting proteins for degradation. Maize pollen was previously reported (Callis and Bedinger 1994) to show extremely low levels of ubiquitin monomer, and developmental significance was attributed to this surprising feature of maize pollen. However, we had previously shown (Muschietti et al. 1994) that tomato pollen had high levels of ubiquitin monomer. Here we show that pollen from most plant families has high levels of ubiquitin monomer. Most grasses tested show reduced levels of ubiquitin monomer, but some maize inbred lines have higher levels of ubiquitin monomer than other inbreds. There was no correlation between the level of ubiquitin monomer and either the monocotyledonous or tri-cellular condition of grass pollen or the dehydrated condition of mature pollen. Since many aspects of pollen development (i.e., wall formation, microspore mitosis, synthesis and storage of mRNAs and proteins, carbohydrates and lipids, dehydration at maturity) are stereotypical among all plant families, the reduced level of ubiquitin monomer in pollen of many grasses cannot be crucial for any feature of normal pollen development.

Key words Ubiquitin · Tomato · Maize · Heat shock proteins · Protein degradation

Introduction

Pollen development is strictly oriented toward its final function, fertilization of the female gametophyte (reviewed in Mascarenhas 1993; McCormick 1993). Pollen has specific and tight control of its gene expression, producing the required proteins at the right time (reviewed in Mascarenhas 1989). For example, pollen genes acti-

vated before microspore mitosis are thought to be required for “early” events in pollen development, such as cytoskeletal organization and pollen cell wall formation (Evans et al. 1992), while “late genes” that are first expressed after microspore mitosis and peak in mature pollen are thought to be involved in pollen maturation or pollen tube growth (Muschietti et al. 1994). While it is reasonable to assume that pollen proteins are degraded after their function has been accomplished, there are no conclusive reports as to whether developmentally controlled protein turnover is important in pollen.

Ubiquitin is a highly conserved 76-amino-acid protein found in all eukaryotic cells and is involved in the most well-characterized protein degradation pathway. Ubiquitin is found free or covalently bound to other cytoplasmic, nuclear or integral membrane proteins. Once multiple ubiquitin moieties are bound, the target protein is rapidly degraded by a protease complex, with the subsequent liberation and reuse of the free ubiquitin (reviewed in Hershko and Ciechanover 1992). Ubiquitin and many ubiquitin genes and ubiquitin pathway enzymes or the genes encoding them have been isolated from plants (reviewed in Vierstra 1993). The patterns of ubiquitinated proteins change during processes such as floral development (Courtney et al. 1994), zygotic embryogenesis (Almoguera et al. 1995), aging and greening of leaves (Veierskov et al. 1992) and seed formation (Ferreira et al. 1995), suggesting that ubiquitin-mediated protein degradation plays a role during all of these processes.

It was recently shown that free and protein-bound ubiquitin levels – but not ubiquitin mRNA – are dramatically reduced during maize pollen development (Callis and Bedinger 1994). The timing of this reduction correlated with the initiation of microspore mitosis and pollen maturation. Because of their finding with maize pollen, Callis and Bedinger suggested that a tight regulation of protein turnover might not be required in mature pollen because it has a short life span. They also proposed that ubiquitination of proteins could be detrimental during pollen dehydration and that ubiquitin protein levels might be down-regulated in order to prevent erro-

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neous interactions between ubiquitin and other proteins that are accumulated to high levels during pollen maturation. In discussing the results of Callis and Bedinger, Worrall and Twell (1994) also proposed that alternative ubiquitin-independent protein degradation pathways, such as transport to vacuoles, might be used in mature pollen.

Although all of these suggestions are provocative, our results (Muschiatti et al. 1994) showed that monoubiquitin is not always so regulated in mature pollen. We had seen no decline in ubiquitin monomer levels in tomato mature pollen when we used it as a protein-loading control in western blots. We therefore examined ubiquitin levels in mature pollen of many plant families in order to address the reason for the differences detected between tomato and maize pollen.

Materials and methods

Plant materials

Maize pollen of a genetic stock tester line from Sarah Hake's laboratory (Plant Gene Expression Center, USDA-ARS/UC Berkeley, Calif.) was used in our initial experiments ('maize' in Figs. 1, 2, 3, 5). The Hake group also provided pollen from tripsacum (*Tripsacum dactyloides*) and coix (*Coix lacryma*), pollen from maize inbreds A188 and B73, and seeds of maize inbreds A188, B73, and Mo17. Pollen from maize inbreds Mo17 and W23 and seeds of W23 were provided by Richard Schneeberger at UC Berkeley. Pollen and seeds of maize inbred A665 were provided by Hank Bass at UC Berkeley.

Spring wheat (*Triticum aestivum* cv. Bob White and cv. Vulcan) pollen samples were provided by Ann Blechl of the USDA-ARS in Albany, Calif. Sorghum (*Sorghum bicolor*) pollen was provided by Jeff Bennetzen of Purdue University. Pearl millet [*Pennisetum glaucum* (L.) R. Br.] pollen was provided by Wayne Hanna of the USDA-ARS in Tifton, Ga. Samples of bamboo (*Pseudosasa japonica*), oxalis (*Oxalis* sp.), rhododendron (*Rhododendron arboreum*) and trumpet vine (*Campsis radicans*) pollen were collected at the UC Berkeley Botanical Garden. Samples of calla lily (*Calla palustris*), cherry (*Prunus cerasus*), and pine (*Pinus* sp.) pollen were collected from plants grown locally. Sunflower (*Helianthus annuus*) and tulip (*Tulipa silvestris*) pollen samples were collected from plants purchased at local nurseries. Pollen of tomato (*Lycopersicon esculentum* cv. VF36) and brassica (*Brassica napus*) was collected at the Plant Gene Expression Center greenhouses.

All pollen samples were stored frozen at -80°C , except for maize inbred pollen used in the experiment shown in Fig. 6, which was collected fresh and used immediately.

Protein extract preparation

Mature pollen samples (10–20 μl) were homogenized on ice in a 0.2 ml capacity ground glass tissue grinder for 1–2 min in 100–200 μl of extraction buffer (50 mM TRIS, pH 7.4/1 mM EDTA/1 mM 2-mercaptoethanol/2 mM pepabloc). After centrifugation at 12 000 g at 4°C for 10 min, supernatant proteins were quantitated by a bicinchoninic acid assay (Pierce). There was no apparent difference in results between samples whose proteins were extracted from frozen pollen and those extracted from fresh pollen.

Proteins were also extracted from embryos dissected from dry maize seeds. Embryos (0.05 g) were ground in a mortar and pestle at room temperature for 1 min, then for an additional 1 min with 1 ml cold extraction buffer. Samples were spun and proteins quantitated as described above.

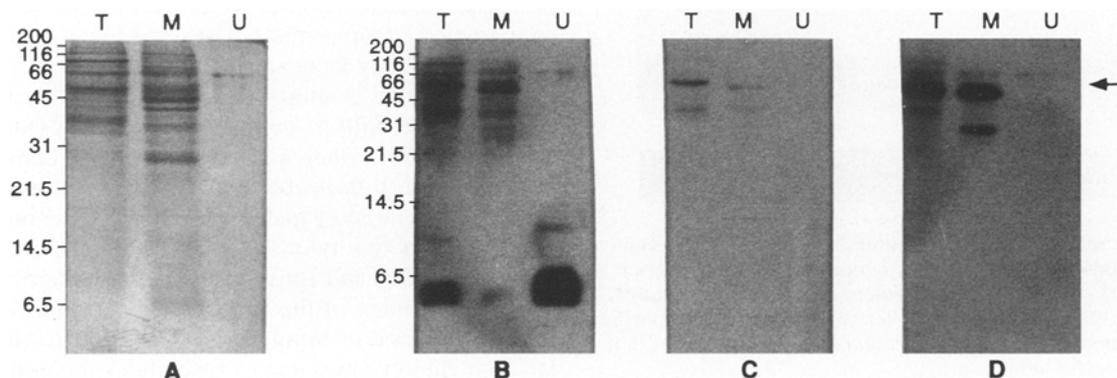
Protein gels and immunodetection

Protein extracts were separated by SDS-PAGE on 15% acrylamide gels (Ausubel et al. 1994). Separated proteins were transferred onto nitrocellulose membranes (Amersham) using the Semi Dry Blotter (E&K). Blots were blocked in 1 \times TBS/0.1% Triton X-100/3% gelatin, and immunodetection was performed as described by Harlow and Lane (1988). Anti-rabbit polyclonal ubiquitin antibodies (Sigma U-5379) and anti-mouse monoclonal α -tubulin antibodies (Amersham NA 934) were diluted 1:1000 in blocking solution. Secondary antibodies linked to horseradish peroxidase were diluted 1:1000 in blocking solution and used with the enhanced chemiluminescence (ECL) system (Amersham).

Results

Because our protein extraction protocol and immunodetection system differ from those used by Callis and Bedinger (1994), it was necessary to repeat the previously reported results in a single western blotting experiment. In our initial experiment (Fig. 1) we compared tomato and maize mature pollen protein extracts on ubiqui-

Fig. 1A–D Ubiquitin detection in maize and tomato pollen. Immunoblot analysis of tomato (*T*) and maize (*M*) mature pollen protein extracts (10 μg each lane) compared to 0.05 μg purified ubiquitin monomer (*U*). **A** Coomassie-stained acrylamide gel. **B** Immunoblot incubated with ubiquitin polyclonal antibody and anti-rabbit IgG secondary antibody. **C** Immunoblot incubated with anti-rabbit IgG secondary antibody alone. **D** Immunoblot incubated with α -tubulin monoclonal antibody and anti-mouse IgG secondary antibodies. The arrow indicates the detection of α -tubulin at 50 kDa



tin immunoblots. In Fig. 1A we show a Coomassie-stained gel demonstrating equal loading of 10 μg each of tomato and maize pollen proteins. The 0.05 μg of purified ubiquitin monomer loaded in the third lane is not visible on this gel. In Fig. 1B we show the ubiquitin immunodetection (using ECL) of a blot with 10 μg each of the same tomato and maize pollen protein extracts as were loaded on the Coomassie-stained gel, and also 0.05 μg purified ubiquitin monomer. It is clear that the tomato sample contains ubiquitin monomer and the maize sample essentially does not, when compared to the lane containing the approximately 5-kDa purified ubiquitin monomer. This initial experiment confirmed our previously published findings that monomeric ubiquitin is present in tomato pollen and confirmed Callis and Bedinger's report that monomeric ubiquitin is absent in mature maize pollen. We did detect some higher molecular weight bands in both samples, which may be ubiquitin-protein conjugates.

To determine whether the higher molecular weight bands detected in Fig. 1B corresponded to ubiquitinated proteins, we tested whether the anti-rabbit IgG secondary antibody cross-reacted with tomato and maize pollen proteins or to purified ubiquitin monomer. The immunoblot incubated with the ubiquitin antibody and the secondary antibody (Fig. 1B), was compared to an immunoblot incubated with the secondary antibody alone (Fig. 1C). The secondary antibody alone does not detect the ubiquitin monomer in either sample. It does detect two larger proteins in each pollen sample, approximately 40 and 55 kDa in tomato, and 40 and 50 kDa in maize. These pairs of bands are thus not ubiquitin-protein conjugates. The additional high molecular weight bands detected may be ubiquitin-conjugated proteins or proteins detected due to cross-reaction with other components of the unpurified polyclonal ubiquitin antibody (Ellison and Hochstrasser 1991). Although Callis and Bedinger

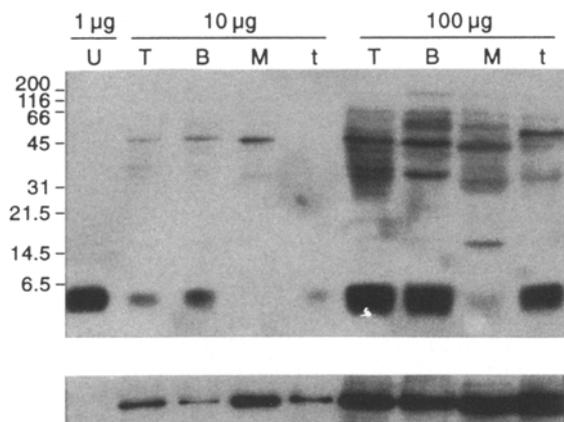


Fig. 2 Comparison of pollen ubiquitin levels in representative monocotyledons and dicotyledons. Ubiquitin immunodetection in 10 μg and 100 μg mature pollen protein extracts from tomato (*T*), brassica (*B*), maize (*M*) and tulip (*t*) compared to 1 μg purified ubiquitin monomer (*U*). The lower panel shows the α -tubulin detection in the same samples

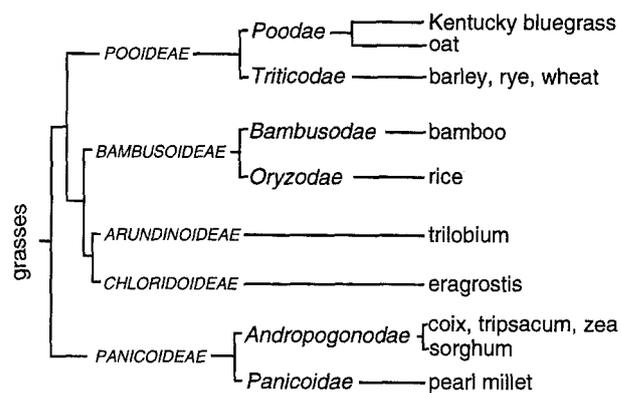


Fig. 3 Grass family phylogeny. Relationships among selected grasses, as arranged by Clayton and Renvoize (1986) and Watson and Dallwitz (1992). The length of lines does not correlate with time or imply any particular evolutionary distance. Subfamily names are *capitalized*, subtribes are *italicized*, and common names are in *lower case*

(1994) used an affinity-purified ubiquitin antibody, we decided to continue using the unpurified antibody. The additional high molecular weight bands detected do not interfere with the interpretation of our results since we concentrate on the ubiquitin monomer in the remaining experiments.

We wondered whether ubiquitin is not detected in maize samples because of inefficient protein extraction. It is possible that we were unable to disrupt the maize pollen cell wall during homogenization. Consequently, maize pollen protein extracts might consist mainly of surface-localized proteins. To address this possibility we re-incubated immunoblots with an antibody to α -tubulin, a known cytoplasmic protein (Fosket and Morejohn 1992) (Fig. 1D). The detection of α -tubulin in the tomato and maize pollen samples indicates that we are successfully extracting cytoplasmic proteins and confirms the ubiquitin detection of both samples; there were high levels of the monomer in tomato, and low or nearly absent levels in maize. The α -tubulin detection is used as a control for all species in all of the following experiments. Although we use a protein quantitation assay in order to load equal amounts of proteins in each lane, the α -tubulin control gives a more accurate description of the amount of cytoplasmic proteins extracted for each sample. By comparing of the ubiquitin signal to the α -tubulin signal of each sample, we can correct for the amount of extracted cytoplasmic (vs surface localized) proteins, which might vary from sample to sample.

Maize pollen is short-lived, a characteristic that is often associated with tri-cellular pollen grains (Stanley and Linskens 1974), whereas tomato pollen is considered to be long-lived, a trait often associated with pollen grains that are bi-cellular at maturity and whose second mitosis gives rise to the two sperm cells during pollen tube growth (Stanley and Linskens 1974). We therefore tested the mature pollen of the following representative species for the presence of ubiquitin protein: tomato (a bi-cellular dicotyledon), brassica (a tri-cellular dicotyledon), tu-

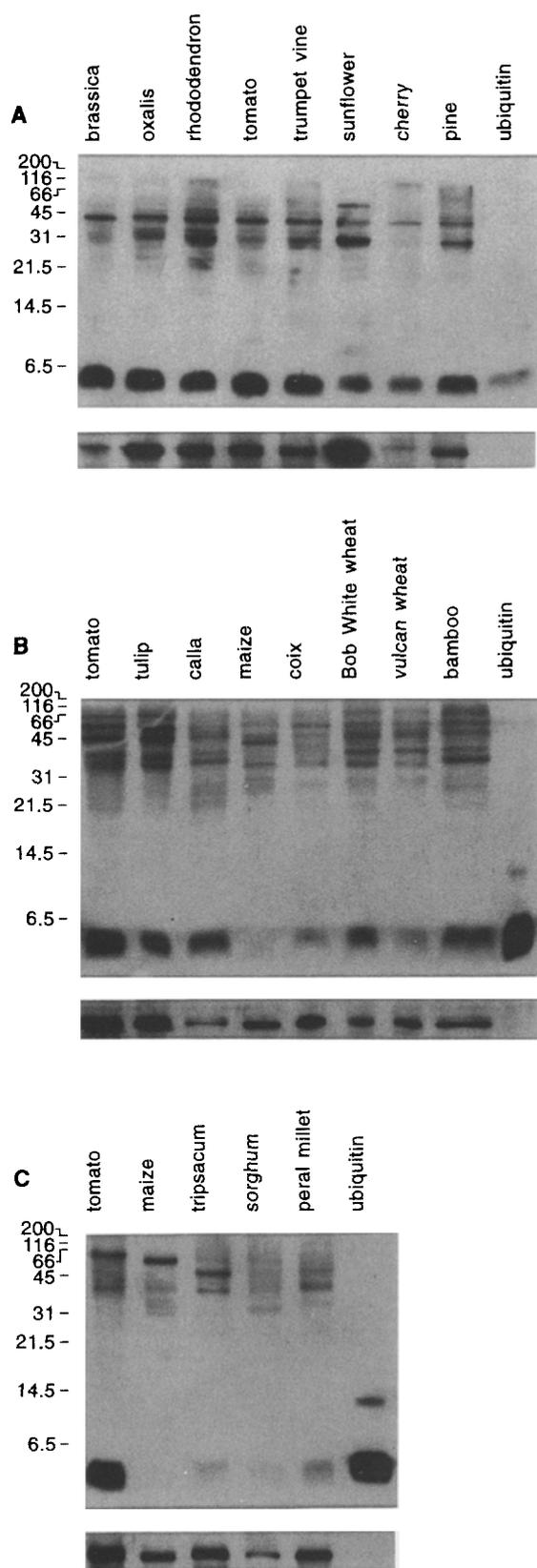


Fig. 4A–C Comparisons of ubiquitin levels in mature pollen of 17 plant species. Ubiquitin immunoblots of mature pollen protein extracts from miscellaneous species (10 μ g each lane) and 0.05 μ g purified ubiquitin monomer. The *lower panels* show the corresponding α -tubulin detection for each immunoblot

lip (a bi-cellular monocotyledon), and maize (a tri-cellular monocotyledon). Figure 2 shows that higher molecular weight proteins, possibly ubiquitin conjugates, were detected in all four species on ubiquitin immunoblots. In lanes containing 10 μ g of pollen proteins, ubiquitin monomer is detected in tomato, brassica, and tulip, but not in maize. This confirmed results reported by Callis and Bedinger (1994) for 10 μ g of maize pollen proteins. However, by loading 100 μ g per lane and using the more sensitive ECL detection system, we are able to detect a small amount of ubiquitin monomer in maize pollen. We estimate that the reduction in maize ubiquitin monomer in comparison to tomato ubiquitin monomer is at least 100-fold.

We sampled additional species to see if the strong reduction in ubiquitin monomer is restricted to maize. Plants were chosen from a wide range of evolutionarily distinct groups (Benson 1979): the Thalamiflorae (brassica and oxalis), the Corolliflorae (rhododendron, tomato, and trumpet vine), the Ovariflorae (sunflower), the Calyciflorae (cherry), and a gymnosperm (pine). Monocotyledons tested included members of the Liliales (tulip), the Arales (calla), and the Graminales (wheat, bamboo, sorghum, pearl millet, coix, tripsacum, and maize). Relationships among some members of the grasses are diagrammed in Fig. 3 (Clayton and Renvoize 1986; Watson and Dallwitz 1992). The immunoblots in Fig. 4 show the results from 17 species tested for the presence or absence of the ubiquitin monomer. Once again, the α -tubulin detection shown below each ubiquitin immunoblot gives an indication of the amount of cytoplasmic proteins in each sample. Notice that in Fig. 4A, the cherry sample seems to have less ubiquitin monomer than the other samples in this blot. However, very little α -tubulin is detected in the cherry sample, indicating poor extraction and underloading of cytoplasmic proteins. Therefore, we reason that cherry actually has ubiquitin monomer levels closer to that of the other dicotyledons. The mature pollen extracts of all of the dicotyledons and pine have the high levels of the ubiquitin monomer shown earlier for tomato. Some monocotyledons (tulip, calla, and bamboo) also show high levels of the ubiquitin monomer, compared to tomato on the same immunoblot. However, coix, wheat, tripsacum, sorghum and pearl millet have much reduced levels of the ubiquitin monomer. Of all the species tested, maize pollen shows the strongest reduction of monomeric ubiquitin.

The maize pollen used in the above experiments is from a genetic tester line, not from Ky21, the inbred line used by Callis and Bedinger (1994). To determine whether the reduction in pollen ubiquitin is uniform within maize varieties, we tested several maize inbred lines for the presence or absence of the ubiquitin monomer (Fig. 5). The maize pollen sample we used for the experiments in Figs. 1–3 was compared to pollen protein extracts of maize inbred lines A188, A665, B73, Mo17 and W23. Lines A188, B73 and Mo17 show the same strong reduction observed previously for the maize genetic stock. However, a significant amount of ubiquitin

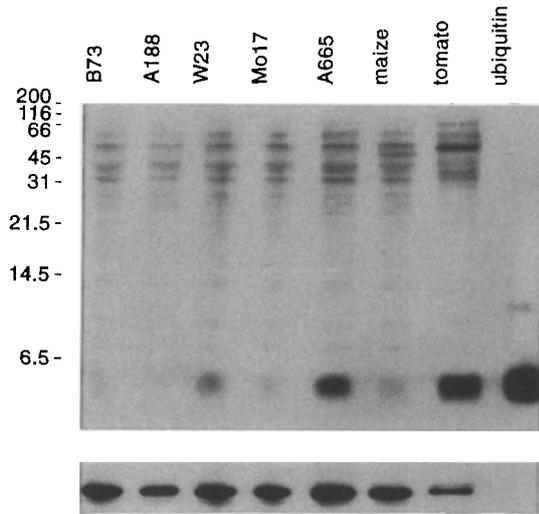


Fig. 5 Comparison of ubiquitin monomer levels among maize inbred lines. Ubiquitin immunoblot of mature pollen extracts from maize inbred lines B73, A188, W23, Mo17 and A665, compared to maize and tomato samples previously shown in Figs. 1–3 (10 µg each sample). The ubiquitin lane contains 0.05 µg purified ubiquitin monomer. The lower panel shows the α -tubulin detection in the same samples

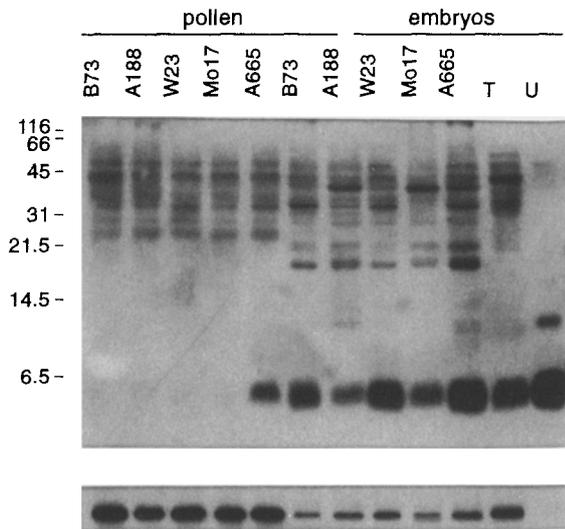


Fig. 6 Comparisons of ubiquitin levels in maize pollen and embryos. Ubiquitin immunodetection in mature pollen and embryo protein extracts of maize inbreds B73, A188, W23, Mo17 and A665 (10 µg each), 10 µg mature pollen protein extracts from tomato (T) and 0.05 µg purified ubiquitin monomer (U)

monomer is detected in W23, and even more in A665. Still, neither maize line has as much ubiquitin as tomato pollen.

We also sought to determine why ubiquitin should be absent in maize pollen, while present in other tissues. Callis and Bedinger (1994) had suggested that, as a result of the drastic dehydration of pollen, new protein conformations or aberrantly interacting proteins might be formed, causing them to be incorrectly targeted for degradation by ubiquitin. For this reason, the ubiquitin-de-

pendent degradation pathway may not be operating in mature pollen. To test whether the reduction of ubiquitin monomer in maize is related to dehydration, we looked at protein extracts of another highly dehydrated tissue: embryos dissected from dry seeds. Pollen protein extracts and embryo protein extracts of maize inbred lines A188, A665, B73, Mo17 and W23 are compared on the ubiquitin immunoblot in Fig. 6. All five embryo samples show high amounts of ubiquitin monomer, similar to levels observed for tomato pollen extracts. Therefore, the level of ubiquitin is not dependent on tissue dehydration alone.

Discussion

We have examined a broad range of plants (one gymnosperm, seven dicotyledons and nine monocotyledons) and have shown that only certain grass species (wheat, pearl millet, sorghum, tripsacum, coix, and maize) show dramatically decreased ubiquitin monomer levels in mature pollen. We attempted to correlate the low levels of ubiquitin monomers to two features of maize pollen with no success; not all monocotyledons (like tulip and calla) and not all tri-cellular pollen (brassica, for example) have low levels of ubiquitin monomer. Pollen grain size was another feature we initially thought might be related to the low levels of ubiquitin monomer. Maize pollen, at 100 µm, is large compared to the 30 µm tomato pollen grain (Stanley and Linskens 1974). However, when we examined the pollen extracts of several maize inbred lines whose pollen grains are essentially the same size, we observed that even some maize lines have significant levels of ubiquitin monomer (Fig. 5). This variation excludes size as an explanation for reduced levels of ubiquitin in maize pollen (and, in fact, some other grass species with low levels of ubiquitin monomer have small pollen). Because we were not able to obtain pollen samples of species in the Arundinoideae or Chloridoideae, and sampled only one plant species in the Bambusoideae, we were not able to determine whether all subfamilies in the grasses have species with drastically reduced levels of ubiquitin monomer. Our examination of dehydrated tissues (mature pollen and embryos from dry seeds in Fig. 6) showed that reduced water content in itself does not prevent accumulation of ubiquitin, since high amounts of ubiquitin monomer were detected in embryos. In fact, ubiquitin monomer levels in embryos were high for all maize inbreds tested, regardless of the amount of ubiquitin in their pollen extracts.

The fact that ubiquitin monomer levels vary among the maize inbred lines suggests that mature pollen ubiquitin levels might also vary within other species if more than one accession is examined. For example, the ubiquitin monomer level varies somewhat between pollen of the two wheat varieties (Fig. 4B). The α -tubulin control shows that approximately equal amounts of cytoplasmic proteins are loaded, but we detect less ubiquitin monomer in pollen from Vulcan wheat than in pollen

from Bob White wheat. We also observed some variation in pollen ubiquitin levels among three tomato accessions when corrected against the α -tubulin control (data not shown). The *L. pennellii* LA716 sample had slightly more ubiquitin monomer than the *L. pennellii* LA2963 sample, and the *L. esculentum* (cv. VF36) sample had levels approximately five times higher than either *L. pennellii* accession. Because maize inbreds show significant variation for the level of ubiquitin monomer, it may be possible to map the locus (or loci) controlling this difference. We plan to test pollen from the inbred parents (Tx303 and Co159, T232 and CM37) that were used to construct recombinant inbred (RI) mapping lines (Burr et al. 1994). If these parents show a difference in the levels of ubiquitin monomer comparable to that seen between the A665 and A188 lines, we will be able to determine the genetic control of ubiquitin levels in mature pollen. If we cannot identify RI parent lines that show such a difference, we can still cross A665 and A188 to determine the inheritance of this trait and then generate an F2 mapping population with these lines.

We know of only two other examples of developmental differences in ubiquitin monomer protein. Ubiquitin protein was detected only in the cortex cells, not in the medulla cells, of the marine sponge *Geodia cydonium* (Pfeifer et al. 1993), although ubiquitin mRNA transcripts are detected in both tissues. Ubiquitin monomer was absent in isolated chloroplasts of spinach and pea, although high molecular weight complexes were detected, suggesting that the ubiquitin conjugates are imported from the cytoplasm to the chloroplasts (Hoffman et al. 1991). However, in *Chlamydomonas* both ubiquitin monomer and ubiquitin conjugates were present in chloroplasts (Wettern et al. 1990).

What can explain the low levels of ubiquitin monomer in mature pollen of certain maize inbreds? A trivial explanation might be that the ubiquitin epitope recognized by the antibody is masked in mature pollen of these inbreds. However, such a difference would have to be developmentally controlled, since earlier developmental stages of Ky21 pollen had detectable ubiquitin monomer (Callis and Bedinger 1994). Another possibility is that maize inbreds vary in the rate of protein turnover in mature pollen. In fact, discussing their work in sponge, Pfeifer et al. (1993) suggested that ubiquitin protein is not detected in medulla cells because protein turnover in medulla cells may be more rapid. Lastly, although the ubiquitin degradation pathway presumably does operate in pollen of some maize inbreds, perhaps another mechanism for protein degradation is operative in pollen of the low ubiquitin inbreds. We plan to investigate whether other components of the ubiquitin-dependent degradation pathway (Vierstra 1993) are affected in the low-ubiquitin monomer maize inbreds. If other ubiquitin pathway components are also down-regulated in pollen of these maize inbreds, it might imply that the genetic difference(s) act at the level of a regulatory gene.

Considering that many maize inbreds do not have high levels of ubiquitin monomer but produce normal

pollen and give a full seed set after self pollination, we can suggest that at least in these inbred lines the presence of ubiquitin monomer is not necessary for normal pollen development. However, it is possible that ubiquitin monomer is required during pollen germination. Thus it may be important to determine whether the ubiquitin monomer levels increase in these inbreds upon pollen hydration and germination. Lastly, we note that mature maize pollen cannot mount a heat shock response to the same extent as can vegetative tissues (Hopf et al. 1992). These experiments were done with the A188 maize inbred line, which, according to our findings, has low levels of ubiquitin monomer in mature pollen. A heat shock response was detected in pollen of the inbred line Mo17 (Frova et al. 1989), which also has low levels of ubiquitin monomer, but these experiments were done with binucleate stage pollen rather than with mature pollen. It will be interesting to test whether inbreds such as A665 that show higher amounts of ubiquitin monomer can mount a more normal heat shock response in pollen. Because heat shock proteins and ubiquitin genes can be induced under other kinds of stress, such as water stress (Borkird et al. 1991), it will also be interesting to find out whether stress treatments can restore ubiquitin monomer to normal levels in pollen of these inbreds.

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